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# THE ROLE OF NEWLY DISCOVERED EXOTOXIN (S TOXIN) IN PSEUDOMONAS AERUGINOSA INFECTIONS

Annual Report

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## Summary

A. Our long term goal is to determine the role of exotoxin S in humans infected with Pseudomonas aeruginosa and develop vaccines to reduce the morbidity and mortality associated with these infections. During the period of the project covered in this annual report (August 1, 1981 - December 31, 1982), we have:

1) Purified two distinct forms of protein S, partially characterized these proteins and continued to characterize the lipid vesicles (found in culture supernatant which contain the S proteins;

2) Prepared specific antisera in rabbits against the two (separate forms of S protein and used these antisera to immunologically characterize the two S proteins and develop a better (more sensitive and specific) assay for S colonies (for mutant and cloning studies);

3) Continued to investigate the role of S in chronic pulmonary infections; most of which were described in last years progress report. Subsequently, we reexamined rat sera with alternative immunological methods and detected S binding antibodies in serum from rats infected with strain 388. We have also begun studies in collaboration with J. Sadoff, testing the efficacy of various P. aeruginosa vaccines in altering or preventing chronic lung infections with P. aeruginosa strain 388 in this rats. The vaccines that have thus far been tested are 2 non-toxic LPS vaccines (1244) and (134VA) and a killed whole cell vaccine of E. coli J-5

4) Continued our genetic studies on S. Ten mutants were isolated, characterized and found unsuitable for virulence studies. Alternative approaches were developed on which our future studies are based.

B. Publications during period covered by this report.

1. Thompson, M.R. and Iglewski. Pseudomonas aeruginosa toxin A and Exoenzyme S in ADP-Ribosylation Reactions, ed. by O. Hayaishi, Academic Press. N.Y. 666, 1982.

2. Woods, D.E., Thompson, M.R., Iglewski, B.H. Demonstrations of Exoenzyme S in Chronic Pulmonary Disease due to Pseudomonas Aeruginosa. Am. Rev. Resp. Dis. in press, 1983.

3. Iglewski, B.H. and Lile, J. Purification and properties of two forms of P. aeruginosa exoenzyme S. Manuscript in preparation, (1983).

Foreword

During the course of this work, the authors were greatly assisted by Dr. M. Thompson (currently at the University of Cincinnatti), Dr. Jay Eisenberg, Mr. Jack Lile and Ms. Cheryl Chaney. Their help is deeply appreciated. Portions of this research were done in collaboration with Dr. J.C. Sadoff, Department of Bacterial Diseases, WRAIR, Washington, D.C. 20012.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animals Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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# I. Purification and Characterization of S Protein(s)

## A. Introduction

We previously reported (annual report dated August 1981) that we had identified two proteins which reacted to our S antiserum (available at that time). Both these proteins were found associated with lipid containing blebs or vesicles. During this contract period, we have purified both of these proteins, and begun to characterize them. Ww have also found that the S containing vesicles appear to be unique from purified outer or inner membranes or envelopes isolated from cells of the S producing strain 388-6.

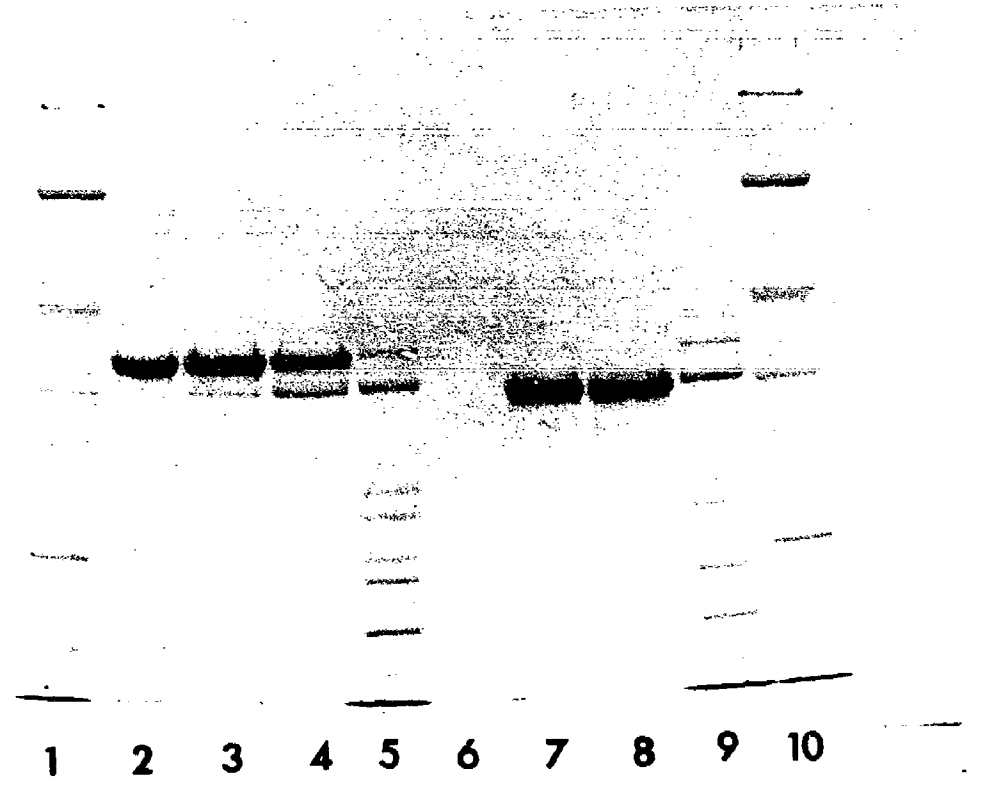
## B. Materials and Methods

The bacterial strain, P. aeruginosa 388-6, the S specific ADP-ribosyltransferase assay, the defined culture medium and culture methods have all been described in detail in previous annual reports and/or published (1-3). Sodium doderyl sulfate (SDS) acylamide gel electrophoresis utilized the procedures of Laemmili (4) with various detergents added as indicated in the results section. Isoelectric focusing was done with pH5-7 ampholites as recommended by Bio Rad.

## C. Results

The S proteins were purified from 2 liters of culture supernatant of strain 388-6 grown in defined medium as previously described (2). The supernatant was filtered thru a 0.2μ cellulose acetate filter to remove residual cells then slowly filtered thru a 0.2u cellulose nitrate filter to which essentially all of the S protein(s) bind. The cellulose nitrate filter is then washed with .50mM tris-10% glycerol-1mM EDTA, pH6.8. The S proteins are then eluted with the same buffer to which has been added 0.2% SDS+0.2%NP-40. This step results in recovery of approximately 80% of the S (enzyme activity present in culture supernatant) and concentrates it 30 fold. As shown in figure 1, lanes 5 and 9, the material eluted from the cellulose nitrate filters contains at least 10-12 proteins distinguishable on SDS-PAGE. The 49,000 molecular weight protein is purified by two sequential preparative slab gel electrophoric steps using the Laemmli-SDS system (4) with 6.5% acrylamide in the separating gel. The first electrophoresis is carried out in the presence of 0.1% SDS. Following electrophoresis, the protein bands are located by overlaying the gel with a sheet of nitrocellulose paper for 1 hour at 25°C. The paper is then stained with amido black and destained to reveal the major protein bands. The stained paper is placed under the gel and the areas under the 49,000 mol. wt. protein is sliced out. The slice is then electroeluted to remove and concentrate the protein. As shown in Figure 1, lane 8, this step eliminates most of the contaminating proteins resulting in primarily the the 49,000 molecular weight protein containing only traces of contaminating proteins. This partially purified 49,000 molecular weight protein is electrophoresed on a second preparative slab gel in the presence of 0.03% NP-40 and 0.01%SDS. The proteins are located, removed and concentrated as described above. As shown in figure 1, lane 7, the resultant 49,000 molecular weight protein appears pure.

Figure 1. Sodium dodecyl sulfate-polyacrylanide gel of S proteins at various steps during purification. Lanes 1 and 10 contain 0.2μgm of a mixture of molecular weight standards. Lanes 5 and 9 each contain 1μgm crude S preparation eluted from a nitrocellulose filter. Lane 8 contains 1.5μgm of the 49,000 molecular weight species of S after the first (0.1% SDS) preparative slab get. Lane 7 contains 1.5μgm 49,000 S protein after the second (0.03% NP40+0.01% SDS) preparative slab ge. lanes 4 contains 1.5μgm of the 53,000 molecular weight protein after the first (0.1% SDS) preparative gel. Lane 3 is the 1.5μgm of 53,000 protein after the second (0.03% NP40+0.01% SDS) preparative gel and lane 2 contains 1.5μgm of the 53,000 protein after the third (0.1% NP40+0.01% SDS) preparative gels. The molecular weight standards in lane 1 are β-galactosidase (130,000); phosphorylase b (93,000) bovine serum albumin (68,000); glutamate dehydrogenase (50,000); ovalbumin (43,000); lactate dehydrogenase (36,000) and carbonic anhydrase (30,000).



The 53,000 molecular weight protein was purified in a similar fashion except it requires three successive preparative electrophoretic steps; The first in the presence of 0.1% SDS; the second in the presence of 0.03% NP40+0.01% SDS and the third in the presence of 0.1% NP 40+0.01% SDS. The protein profiles obtained after each step in the purification of the 53,000 mol. wt. protein and shown in figure 1, lines 2-4. As seen in line 2, figure 1, these procedures result in a pure preparation of the 53,000 mol. wt. protein. These procedures yield approximately 250μgm of the 49,000 and 200μgm of the 53,000 mol. wt. S proteins per 2 liters of starting material. Unfortunately, numerous attempts to scale up these procedures have failed. However, the procedure, as described, though labor intensive, will provide sufficient materials for our future planned studies. The purity of these two proteins was further assessed by subjecting each to SDS and non-denaturing PAGE under several different conditions (pH and acylamide concentrations). In each case, the proteins appeared homogenous. The purified proteins were also isoelectric focused in pH 5-7 ampholytes. Each preparation gave a single sharp band. The pKI of the 49,000 mol. wt. protein was 6.05 and that of the 53,000 mol. wt. protein was 5.90

We have compared the ADP-ribosyltransferase activity of the purified 49,000 and 53,000 molecular weight proteins using wheat germ extract as a source of substrate proteins as previously described (1,2). As shown in Table 1, the 53,000 molecular weight protein is virtually devoid of enzymatic activity as compared to the 49,000 molecular weight protein.

Table 1. ADP-ribosyltransferase activity of purified S proteins[1]

| Protein | p moles $^{14}C$ ADP-ribose transfered/μgm S protein[2] |
|---|---|
| 49,000 | 25,000 ± 150[2] |
| 53,000 | 1.66 ± 0.2[2] |

1. Assay previously described (2)
2. Average of three separate assays ± SEM.

We have also tested the mouse toxicity of crude S proteins (culture supernatant) and the purified 49,000 and 53,000 forms of S proteins. The results shown in Table 2 indicate that up to 3μgm of either purified S protein or a mixutre containing 1.5μgm of each is not toxic to mice. It is perhaps not surprising that these purified S proteins are nontoxic since the purification procedures are far from gentle and involve subjecting the proteins to considerable amounts of detergents which could have destroyed their toxicity. We also tested the toxicity of crude S (cultured supernatant). Amounts containing approximately 1.5μgm of each of the 2 forms of S (53,000 and 49,000) killed 6/6 mice within 48 hours. Since this is crude material and contains LPS and many other proteins, these results must be interpreted cautiously. We are currently attempting to neutralize the mouse toxicity of the crude S with the

specific antisera (described below) raised against the purified 49,000 and 53,000 mol. wt. proteins.

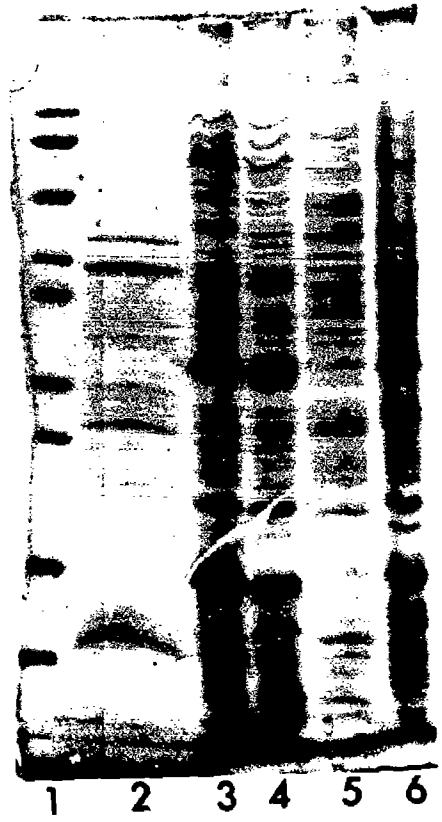
Table 2. Mouse Toxicity of Crude and Purified S. Proteins[1]

| Reagent | Amount (μgm) Injected IP | # Mice Dead/Total Mice |
|---|---|---|
| 49,000 protein | 0.3 | 0/6 |
| | 1.0 | 0/6 |
| | 3.0 | 0/6 |
| 53,000 protein | 0.3 | 0/6 |
| | 1.0 | 0/6 |
| | 3.0 | 0/6 |
| 49,000 + 53,000 | 0.3 (of each S protein) | 0/6 |
| 49,000 + 53,000 | 1.0 (of each S protein) | 0/6 |
| Crude S[2] | Contains approximately 1.5μgm 49,000 +53,000 proteins | 6/6 (within 48 hrs) |

1. Animals observed for 6 days.
2. Filter sterilized culture supernatant of strain 388-6 (S+, toxin A$^-$ protease$^-$) grown in defined medium.

As reported in last years annual report, S appears to be associated with vesicles in the culture medium. Gonkema, et. al. (5) reported that most of the heat-labile enterotoxin (LT) released by E. coli strain AP1 is associated with medium vesicles which closly resemble the outer membrane of this E. coli strain. We therefore isolated and compared the proteins of the S containing vesicles with protein profiles of outer membranes (OMP) cytoplasmic membranes (CMP) and envelopes (EP) prepared from strain 388-6. The membranes and envelopes were isolated and purified as described by Hancock and Nikaida (6). As shown in figure 2, the SDS-PAGE protein profile of medium vesicles containing both the 49,000 and 53,000 molecular weight forms of S do not resemble the protein profiles of OMP, CP or EP. These S containing vesicles are not, therefore, just shed outer membrane or if they are they contain only discret parts of the membrane which lack the major outer membrane proteins.

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel profile of proteins from S containing vesicles (lane 2 [μgm protein]) compared to 25μgm purified outer membranes (lanes 3 and 4), 25μgm purified cytoplasmic membranes (lane 5) and 25μgm cell envelopes (lane 6) isolated from P. aeruginosa strain 388-6 grown in defined medium (2). Lane 1 contains molecular weight standards including β galactosidase (130,000); phosphorylase (93,000); bovine serum albumin (68,000), glutamate dehydrogenase (50,000), ovalbumin (43,000); lactate dehydrogenase (36,000); carbonic anhydrase (30,000); soybean trypsin (21,000) and lysozyme (14,000).



## II. Production and Characterization of Antiserum to Purified S Proteins

### A. Introduction

In order to further investigate the relationship of the 53,000 and 49,000 molecular weight forms of S and develop reagents for future studies, we produced antiserum to each of these purified proteins.

### B. Materials and Methods

The 49,000 and 53,000 molecular weight S proteins were purified as described above (Section I). Three rabbits (New Zealand) were injected with 100μgm of either the purified 49,000 or 53,000 molecular weight S protein suspended in an equal volume of Freund's complete adjuvant. The antigen was injected subcutaneously, intramuscularly and in the rear foot pads. Four weeks later, the rabbits were boosted with 100μgm of the same protein suspended in Freund's incomplete adjuvant. Serum was collected 2 weeks later and tested for enzyme neutralyzing activity and precipitating antibody in immunodiffusion assays as previously described (2).

### C. Results and Discussion

The rabbits immunized with the 49,000 mol. wt. protein had enzyme neutralizing titers of 1/8, 1/16 and 1/32. The serum from the rabbits immunized with the (enzymatically inactive) 53,000 molecular weight protein had enzyme neutralizing titers (against the 49,000 mol. wt. enzyme) of 1/4, 1/32 and 1/64. Thus, both proteins induced antibodies which neutralized the ADP ribosyltransferase activity of S. Similarly, both types of antiserum neutralized the ADP-ribosyltransferase activity of crude S.

All 6 antiserum contained S precipitating antibodies. Typical immunodiffusion patterns obtained with these antisera against the purified proteins are shown in figures 3 and 4. As seen in figure 3, antiserum against the 49,000 molecular weight protein reacted with both the 53,000 and 49,000 mol. wt. proteins and gave a line of identitiy. As shown in figure 4, the antiserum raised against the 53,000 mol. wt. protein also reacted with both the 49,000 and 53,000 molecular weight proteins, however, a reaction of only partial identity is seen with an apparent spur in the direction of the 53,000 mol. wt. protein. This indicates that the larger protein has immunodeterminants not present on the 49,0900 mol. wt. protein. Alternatively, one might argue that these protiens were not totally pure and contained traces of each other. This is complicated by the fact that the 49,000 molecular weight protein tends to break down or aggregate in the gel diffusion system and frequently gives 2 lines against either antiserum. While we cannot rule out minor contamination of our antigens with polyclonal antisera, it does, however, seem unlikely.

These data support the conclusion that the 53,000 and 49,000 mol. wt. proteins are different forms of S, the larger being a proenzyme form and the smaller the enzymatically active form. Conclusive evidence for this will require monoclonal antibodies, genetic analysis, or conversion of the 53,000 inactive ADP-ribosylatransferase form to the 49,000 active

form in vitro. We have tried (unsuccessfully) a number of methods (proteolysis; urea + SDS and mild heating) to convert the 53,000 protein to the 49,000 form.

Figure 3. Gel immunodiffusion pattern comparing reactivities of the two different S proteins against rabbit antiserum raised against the 49,000 molecular weight form of S. The center well (A) contains a 1:2 dilution of antiserum (anti 49K). Wells number 1 contains 1µgm pure 49,000 protein, 2 contains 1µgm 53,000 protein, 4 contains 0.5µgm 49,000 protein and 5 contains 1µgm 53,000 protein. Wells 3 and 6 are empty.

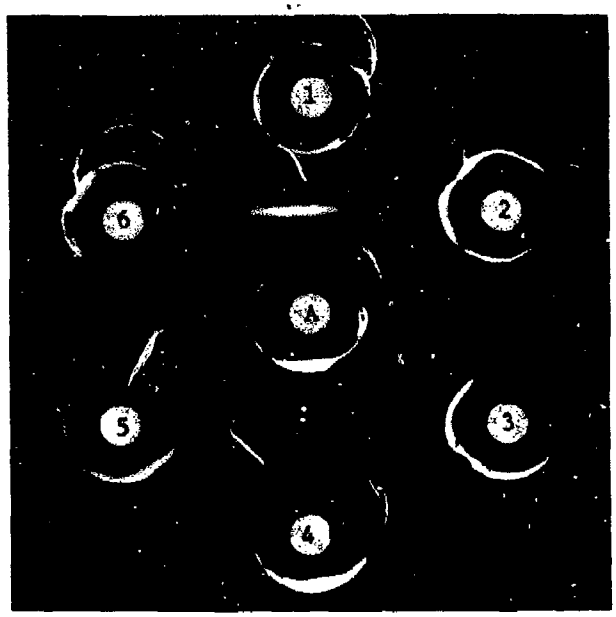
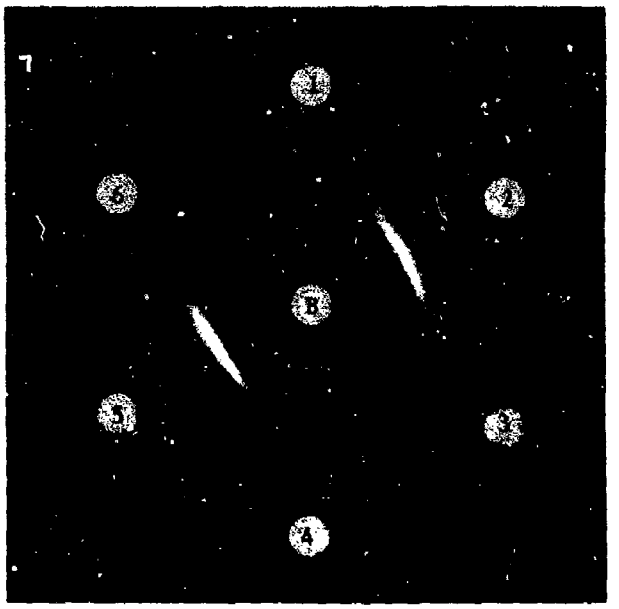


Figure 4. Gel immunodiffusion patterns comparing reactivities of the two different S proteins against rabbit antiserum raised against the 53,000 molecular weight form of S. The center well (B) contains a 1:2 dilution of antiserum (anti 53K). Well number 1 contains 1µgm pure 49,000 protein, 2 contains 1µgm 53,000 protein, 4 contains 0.5µgm 49,000 protein and 5 contains 1µgm 53,000 protein. Wells 3 and 6 are empty.



## III. Chronic Lung Infections with the S Producing Strain (388) of P. aeruginosa

### A. Introduction

We have re-examined serum from rats chronically infected with strain 388 ($S^+$) for 30 days for the presence of S antibodies. We have utilized this animal model of chronic lung infections by P. aeruginosa to investigate the efficacy of three expermental P. aeruginosa vaccines and to form the basis for our future studies with S vaccines.

### B. Methods and Materials

The chronic lung infection model in rats was as previously described (annual report dated August 1981, and as discussed in the manuscript appended - Woods, et. al., 1983). Bacteriological strains, growth conditions, bacterial quantitation and pathology are also described in the appended manuscript. Protein S binding antibody in rat serum was detected by Western blot analysis as described by Towbin, et. al. (7). The three experimental vaccines were obtained from Dr. J. Sadoff (Department of Bacterial Diseases, WRAIR). These included detoxified preparations of LPS from a serogroup I strain of P. aeruginosa (vaccine = 134VA) and detoxified LPS from a serogroup 6 strain of P. aeruginosa (vaccine = 1244) and a whole cell killed preparation of E. coli J-5 (vaccine = J5). Each experimental group contained 20 rats. Group 1 was unimmunized (controls). Group 2 received 10µgm (in 0.1 ml) subcutaneously of vaccine 134VA on days 0, 14 and 21. Group 3 rats were similarly immunized except with 10µgm of vaccine 1244 on each vaccination day. Group 4 received 0.2 ml (equals $5 \times 10^7$ cells) of J5 vaccine subcutaneously on days 0, 14, and 21. Animals were prebled before the first immunization and again on day 28 (7 days after the last injection). On day 28, the right lung of each rat was infected with P. aeruginosa (strain 388 is a serotype 6) cells immeshed in agar as previously described (8 and Woods, et. al. 1983, appended). Six rats/group were bled then sacraficed at 3, 9 and 30 days post infection. The right lungs from 2 rats/group were processed for pathology and those from 4/group processed for bacteriology. Antibody titers are currently being determined in ELISA assays specific for type 1 or 6 P. aeruginosa LPS. Serum from rats immunized with J5 will be analyzed by Dr. E. Ziegler, San Diego, for J5 antibodies

### C. Results and Discussion

Previously (annual report dated August 1981) we described the use of the Cash model (8) to evaluate S and S producing strains in chronic lung infections employing rats. We were surprised that although we could demonstrate S protein in the lungs of infected rats we could not detect S neutralizing antibody in the sera of these rats. We re-examined these sera and in 7/7 rats infected for at least 30 days with stain 388, S binding antibody was detected. Our study has been re-writted and is currently in press. (Woods, et. al., manuscript attached).

We wish to utilize this model as one in which to evaluate S vaccines while at the same time making it available to other investigators to

evaluate their experimental P. aeruginosa vaccines. Three experimental P. aeruginosa vaccines (from Dr. J. Sadoff) are currently being evaluated by us in this model. The results, while incomplete, are encouraging. The number of bacteria recovered from animals infected with strain 388 ($S^+$)and a serogroup 6 LPS type) on days 3, 9, 30, (post infection) is somewhat variable (Table 3). We and the others (8,9) have observed this before. However, the data (Table 3) suggests that the detoxified type 6 LPS vaccine 1244 is protective in that only 3/12 of rats immunized with 1249 had $>10^2$ bacteria/lung recovered over the 30 day experimental period. Some protection was also seen with the E. coli J5 vaccine but to a lesser extent. As expected, the heterologous LPS vaccine 134VA had no effect on lung colonization with strain 388. These results are preliminary but encouraging. The pathology seen in lungs from these four different groups did not vary greatly. The histopathology from all rats examined (24 total) showed seven parenchymal and bronchial changes at all sample times similar to those reported by Woods, et. al. (manuscript appended). Unfortunately, the antibody studies (which are being processed in a blind fashion) are not completed. It seems pointless to try and draw conclusions at this time without the immunological data. These results are, however, encouraging and we intend to test immunization with purified S protein alone and together with vaccine 1244 or J5 in this model this coming year.

Table 3. Effect of Immunization with Experimental LPS Vaccines on P. aeruginosa Growth in Rat Lungs[1]

| | Colony-Forming Units[2] Days Post Infection | | |
|---|---|---|---|
| Vaccine Group | 3 | 9 | 30 |
| Unimmunized Controls | $1.5 \pm 1.2 \times 10^6$ (4/4)[3] | $2.2 \pm 0.6 \times 10^7$ (3/4) | $6.6 \pm .8 \times 10^5$ (3/4) |
| 134VA (LPS Type 1) | $1.3 \pm .8 \times 10^8$ (3/4) | $1.1 \pm 0.8 \times 10^6$ (3/4) | $4.5 \pm 9 \times 10^8$ (4/4) |
| 1244 (LPS type 6) | $3.2 \times 10^8$ (1/4) | $9.4 \times 10^5$ (2/4) | NG[4] 0/4 |
| E. coli J5 | $8.3 \pm .4 \times 10^6$ (3/4) | $4.4 \pm 1 \times 10^7$ (3/4) | NG 0/4 |

1. Right lungs of rats infected on day 0 with P. aeruginosa strain 388 in agar beads as described (Woods, et. al., appendix).

2. Colony forming units (CFU) are mean of CFU from bacteria$^+$ rats ± SEM. Four rats were in each group. Our procedure, however, fails to detect $<10^2$ CFU/lung which are considered as negative.

3. The numbers in parenthesis represent the numbers of rats with no detectable CFU/total rats in the group.

4. No detectable growth.

## IV. Genetic Experiments

### Introduction

We have continued our genetic studies on protein S. We found N-methyl-N'nitro-N-nitrosoguanidine (NG) unsuitable for isolating S specific mutants. We developed alternative approaches to be used in future studies.

### Methods and Materials

Stain 388, media, growth conditions, the Welek assay for S and the ADP-ribosyltransferase assay were as described in previous annual reports. Other assays used to characterize mutants were as previously described (10, 11).

An improved filter blot assay employing S specific antisera was developed. This method is described in detail in this years contract renewal application in section D1. Ethyl methyl sulfonate (EMS) was employed as described by Carlton and Brown (12). A broad temperature sensitive host range plasmid derived from RP4 (pTH10) containing TN1 was obtained from Dr. R. Hancock and transfered from strain PA05 to strain 388 via conjugation employing simple plate mating as previously described (13).

### Results

A total of 50,000 colonies were screened by our S specific Welek assay following 4 separate NG mutagenesis of strain 388. Twenty five presumptive $S^-$ mutants were identified of which 10 were stable and remained Welex negative after recloning. These 10 mutants and the parent strain 388 were grown in 10ml of defined medium under conditions optimum for S production (2). The amount of S produced by each mutant strain was compared to the parent in the ADP-ribosyltransferase assay (3). Seven of the mutants produced between 1-10% of the parental levels of S. Whereas, 3 mutants produced less than 1% of the parental level of S. These latter 3 $S^-$ mutants (388-2, 388-6 and 388-9) were further characterized. Cell associated S levels were measured (in the enzyme assay) following lysis of the cells in a French press. Approximately 12% of the total S produced by the parental strain 388 is cell associated. The mutant cells had <1% of the cell associated S activity as the parent but the ratio of extracellular to cell associated S activity was the same with these mutants. This indicates these mutants do not accumulate S intracellularly. Upon further analysis, mutant strains 388-2 and 388-9 were found to be deficient in production of elastase and mutant 388-6 was deficient in production of elastase and alkaline protease. Thus, of the 3 sever $S^-$ mutants, all 3 are pleotrophic and unsuitable for animal studies.

Alternative approaches to use with strain 388 were devised. First; we have constructed a strain of 388 (388-pTH10) containing a temperature sensitive derivative of the plasmid RP4 (pTH10). Strain 388-pTH10, when grown at 30°C is resistant to kanamycin ($Km^R$),tetracycline ($tet^R$) and ampicillin ($am^R$). When we shift the growth temperature up to 42°C only 1

out of $10^4$ cells grows on medium contaiing these antibiotics. In this case, the plasmid has presumably been integrated into the chromosome of strain 388 (14). Each cell which grows at 42°C. in the presence of the appropriate antibiotic should represent a mutant. We intend to screen these cells for $S^-$ and $S^{CRM}$ mutants in the future. We will also manipulate these mutants to cause excision of the plasmid but retention of the transposon. Such mutants will be useful in cloning S genes and for animal studies.

In other studies in my laboratory, we have utilized recombinant DNA techniques to clone the genes encoding P. aeruginosa elastase and (separately) P. aeruginosa alkaline protease. These techniques and reagents will be useful in our future studies of S.

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